NCI Alliance for Nanotechnology in Cancer

Physicochemical Characterization of Nanomedicines: Characterization Considerations and Challenges

Jeffrey D. Clogston, PhD

Principal Scientist, PCC Section Head

ncl@mail.nih.gov

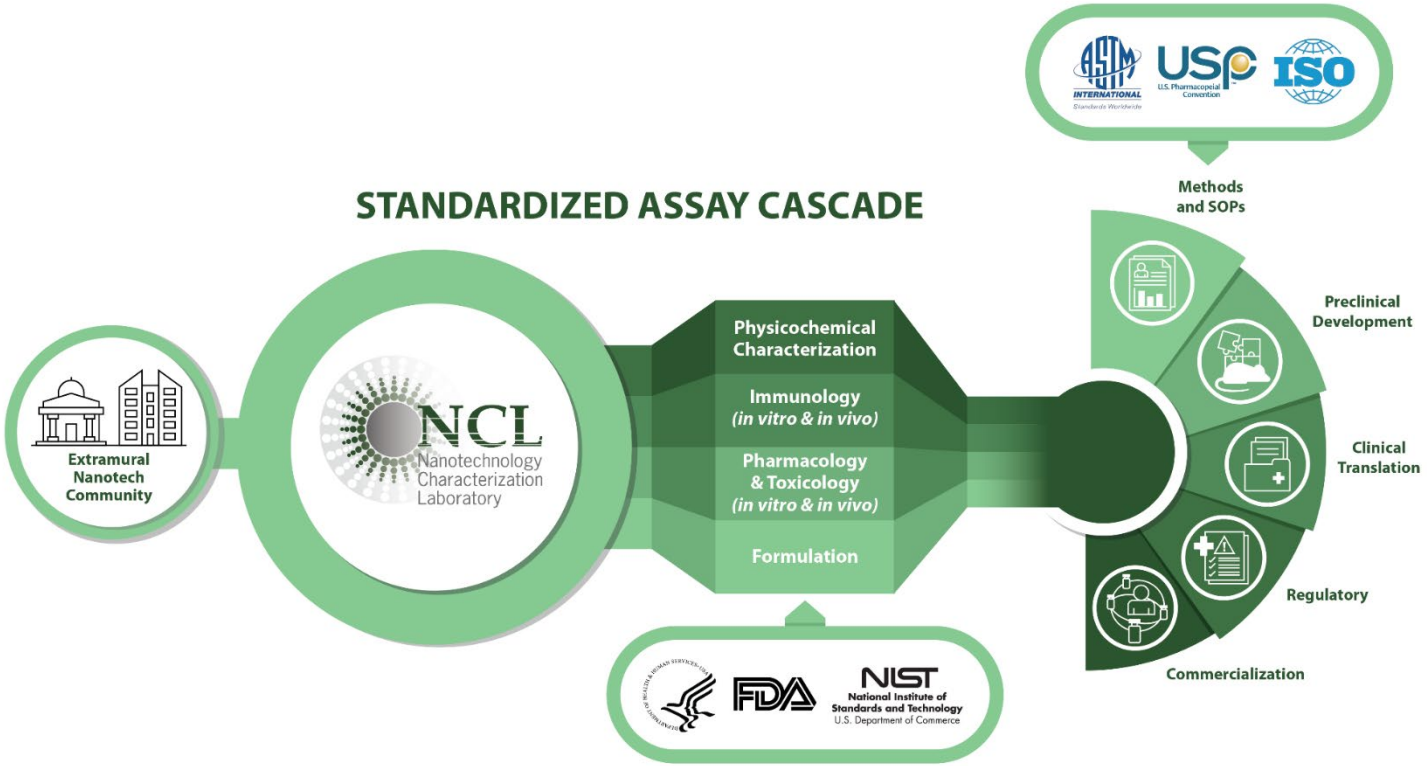
https://ncl.cancer.gov

Frederick National Laboratory for Cancer Research

Nanotechnology Characterization Lab (NCL)

NCL was established in 2004 as a collaboration among the NCI, NIST and FDA, with 4 primary objectives:

- Characterize nanoparticles using standardized methods
- Facilitate regulatory review of nanotech constructs
- Conduct structure activity relationship (SAR) studies
- Engage in educational and knowledge sharing efforts



Assay Cascade is a free service

NCL has 15+ years of knowledge and expertise in nanoparticle characterization and utilizes this to *help accelerate the translation of promising nanotech drugs and diagnostics.*

Visit https://ncl.cancer.gov/

https://www.cancer.gov/nano/research/ncl/assay-cascade/application-process

NCL Assay Cascade – 70+ Standardized Protocols for Nanotech

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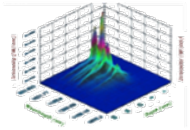

Physicochemical Characterization

Size/Size Distribution
- Dynamic Light Scattering (DLS)
- Electron Microscopy (TEM, SEM, cryo)
- Atomic Force Microscopy (AFM)
- Field Flow Fractionation (FFF), SEC-MALLS

Composition
- TEM with EDS
- Inductively coupled plasma-mass spec. (ICP-MS)
- Spectroscopy (NMR, CD, Fluorescence, IR, UV-vis)

Purity
- Chromatography
- Capillary Electrophoresis

Surface Chemistry
- Biacore
- Zeta Potential

Stability
- Stability can be measured with any number of instruments with respect to time, temperature, pH, etc.

In Vitro Characterization

Sterility
- Bacterial/Viral/Mycoplasma
- Endotoxin

Cell Uptake/Distribution
- Cell Binding/Internalization
- Targeting

Hematology
- Hemolysis
- Platelet Aggregation
- Coagulation
- Complement Activation
- Plasma Protein Binding

Immune Cell Function
- Cytokine Induction
- Chemotaxis
- Phagocytosis
- Leukocyte Proliferation
- Leukocyte Procoagulant Activity

Toxicity
- Cytotoxicity
- Autophagy

In Vivo Characterization

Pharmacology
- Clinical Tx cycle
- NP Quantitation methods
- PK Parameters

Immunotoxicity
- Local lymph node proliferation assay
- T-cell dependent antibody response
- Adjuvanticity
- Rabbit pyrogen test

Single and Repeat Dose Toxicity
- Blood Chemistry
- Hematology
- Histopathology (42 tissues)
- Gross Pathology
- Immunogenicity

Efficacy
- Therapeutic
- Imaging

NCL testing links physicochemical properties to biological outcomes.

70+ protocols available online: https://www.cancer.gov/nano/research/ncl/protocols-capabilities

NCL Collaborators in Clinical Trials

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NCL Supports:

- Preclinical Characterization
- Regulatory Concerns
- Clinical Characterization
- Exploring Alternate Indications
- Next-Generation Nanoparticles

17 Collaborators in clinical trials with novel nanomedicine therapies.

Visit https://ncl.cancer.gov/

Physicochemical Characterization

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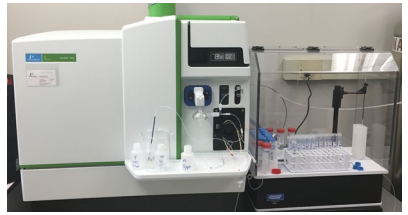
Physicochemical characterization boils down to analytical instrumentation and development of new methods

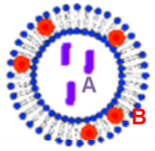
- Dynamic Light Scattering (DLS)
- Static Light Scattering (MALS)
- Laser Diffraction
- Electron Microscopy (TEM, SEM, cryo-TEM, EDS)
- Atomic Force Microscopy (AFM)
- Resistive Pulse Sensing (RPS)
- Zeta Potential

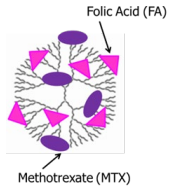


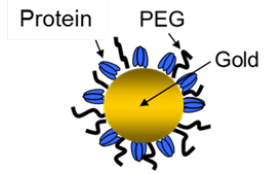
- Chromatography (RP-HPLC, SEC, AF4, FPLC)
- Liquid chromatography–mass spectrometry (LC-MS)
- Gas chromatography–mass spectrometry (GC-MS)
- Inductively coupled plasma-mass spectrometry (ICP-MS)
- CHNOS Elemental Analysis
- Spectroscopy (UV-Vis, Fluorescence, IR, Raman)
- Thermal Analysis (TGA, DSC)
- Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

Leveraging over 16 years of experience, NCL has identified critical quality attributes (CQAs) and methodology needed to support the most common nanoparticle platforms used.

- Liposomal Products
- Polymeric Nanoparticles
- Colloidal Metal Nanoparticles







Capabilities & Instrumentation: https://www.cancer.gov/nano/research/ncl/protocols-capabilities

Parameters, Methods & Considerations for the Physicochemical Characterization of Liposomal Products

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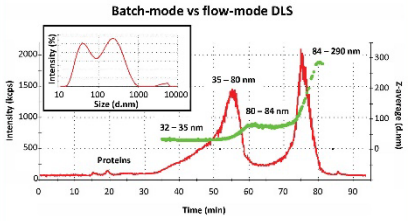
Size/Size Distribution

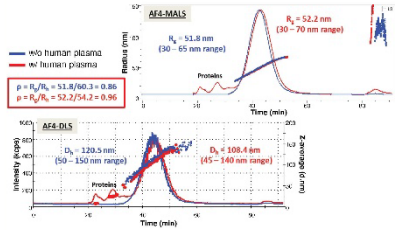
- Dynamic light scattering (DLS)
- Multi-angle light scattering (MALS)
- Laser diffraction
- Cryogenic-transmission electron microscopy (Cryo-TEM)
- Resistive pulse sensing
- Asymmetric-flow field-flow fractionation (AF4) – MALS/DLS



Batch-mode (inset) versus flow-mode DLS measurements of dual-drug loaded liposomes. Multiple size populations are observed by both techniques and indicate a polydispersed sample. However, flow-mode DLS can better resolve the size distribution of each population. Adapted from Anal Bioanal Chem, 2020, 412(2), 425-428.

Surface Characteristics

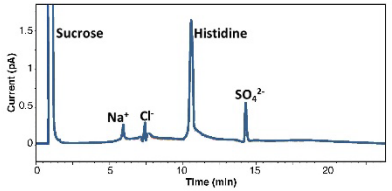
- Zeta potential
- Protein binding assessment by AF4-MALS/DLS
- Quartz crystal microbalance with dissipation monitoring (QCM-D)



Flow-mode AF4-MALS (top) and AF4-DLS (bottom) of PEGylated irinotecan liposomes before and after incubation in human plasma. The increase in the ratio (ρ) of the measured MALS (R_g) and DLS (R_h) sizes after human plasma incubation suggests protein binding to the surface of the liposomes. Adapted from Anal Bioanal Chem, 2020, 412(2), 425-428.

Composition

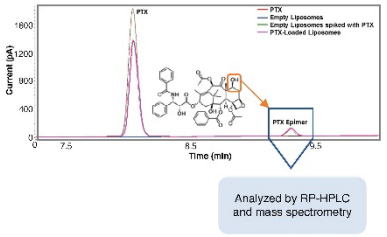
- Drug concentration: total, free & encapsulated
- Drug distribution as a function of size
- Targeting ligand concentration: total, bound & unbound
- Individual lipid concentrations
- Counterion concentrations: interior & exterior
- Excipient concentrations
- Particles per mL concentration
- Osmolality, viscosity measurements



Counterion and excipient concentrations for PEGylated liposomal doxorubicin measured by RP-HPLC with charged aerosol detection (CAD). Adapted from J Pharm Biomed Anal, 2019, 165, 41-46.

Purity

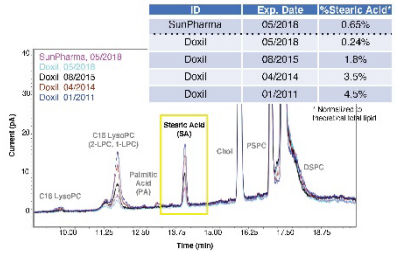
- Drug impurities
- Lipid impurities
- Free drug/lipid/targeting ligand concentrations
- Residual solvents and reagents



Purity assessment of PEGylated liposomal paclitaxel (PTX) as defined by the presence of drug impurities. The drug epimer concentration was measured by RP-HPLC and its identity confirmed by mass spectrometry.

Stability

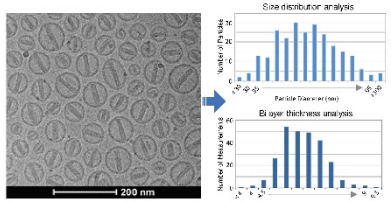
- Size/Size distribution; aggregation
- Drug leakage and degradation
- Hydrolysis of lipids
- Drug release in plasma
- Solvent, thermal, pH, photo, freeze-thaw, lyophilization, centrifugation, filtration
- Storage conditions/shelf-life



Stability assessment of PEGylated liposomal doxorubicin as defined by the hydrolysis of phospholipids. The formation of free fatty acids and lysophospholipids of several batches with varying expiration dates were measured by RP-HPLC with charged aerosol detection (CAD).

Morphology

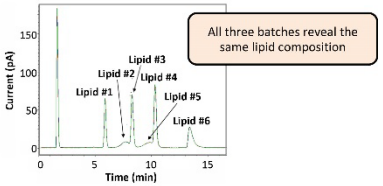
- Cryo-TEM can be used to evaluate:
  - Size distribution
  - Liposome morphology
  - Internal liposome volume
  - Bilayer thickness
  - Number of lamellae
  - Drug appearance/state



Representative cryo-TEM image of PEGylated liposomal doxorubicin. Cryo-TEM was used to determine size and morphology of the liposomes.

Batch-to-Batch Consistency

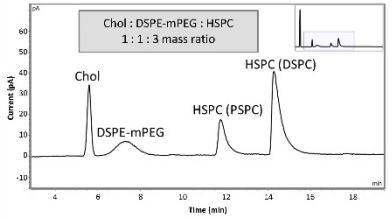
- Assessed by choosing relevant parameters (i.e., lot release criteria) that relate to a desired in vivo outcome



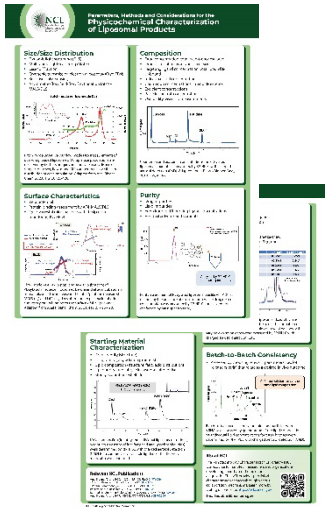
Batch-to-batch consistency for lipid nanoparticles with siRNA was assessed by quantitation of the lipid composition. Six individual lipid concentrations for three batches were determined by RP-HPLC with charged aerosol detection (CAD).

Starting Material Characterization

- Drug identity (structure)
- Drug purity (degradation products)
- Lipid composition (structure, fatty acid distribution)
- Lipid purity (free fatty acid, lysophospholipids)
- Storage conditions/shelf life



Lipid composition (identity and individual lipid concentrations) and purity (presence of free fatty acid and lysophospholipids) were determined by RP-HPLC with charged aerosol detection (CAD) for a commercially available lipid mix. The theoretical mass ratio was confirmed.



Handout available for download on NCL website

Handouts for Polymeric Nanoparticles and Colloidal Metal Nanoparticles also available for download on NCL website

https://www.cancer.gov/nano/research/ncl/protocols-capabilities/physicochemical-characterization-liposomal-products.pdf

Ideally…

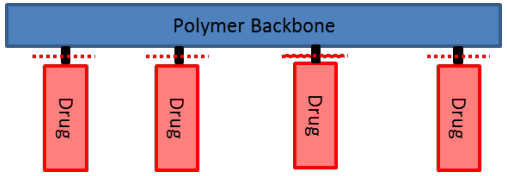
- Critical quality attributes (CQAs) are known and defined for the nanoformulation
- The methodology to measure these CQAs are developed and optimized
- The associated analytical techniques and instrumentation are available and validated
- Reference standards are also available

Drug Loading Quantification of Prodrugs

- **Drug loading is one of the most important critical quality attributes (CQAs) of prodrugs.**
- **Quantification of chemically conjugated drugs in polymeric prodrugs is difficult.**
  - ➢ **Development of novel orthogonal method**

Polymer with Conjugated Drug

- Drug absorbs at a unique wavelength but UV-Vis detection not sensitive enough
- Wavelength shift observed for conjugated drug
- Chemical method needed

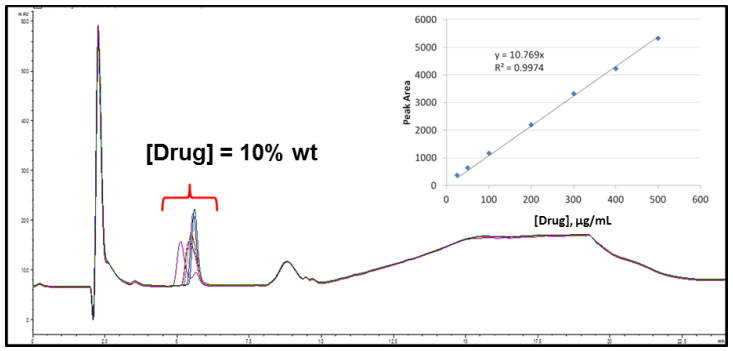


Hydrolysis Method

1) 20 µL sample (in 50 %(v/v) ACN) + 20 µL 1 M NaOH.
2) Incubate overnight at room temperature.
3) Add 20 µL 1 M HCl to neutralize.

followed by RP-HPLC separation

4) Assayed by RP-HPLC with UV detection

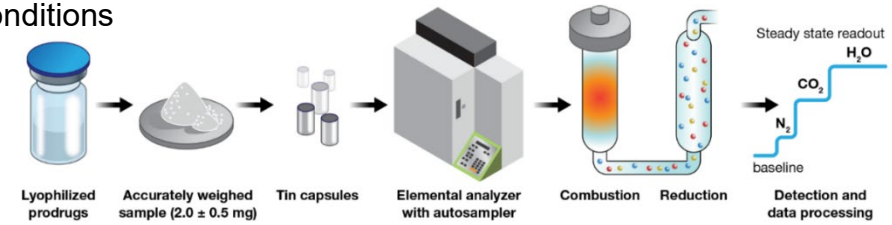


Drawbacks

- Chemical method optimization
- HPLC to separate components

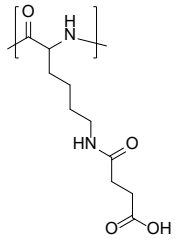
Elemental analyzer – the application of combustion analysis

- Determine the elemental composition by combusting the sample under certain conditions
- Only elements of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) as combustion of materials are used to convert to their oxidized form (CO_2, H_2O, NO or NO_2, and SO_2) under high temperature high oxygen conditions

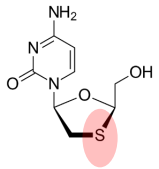


http://www.perkinelmer.com/product/2400-chns-o-series-ii-system-100v-n2410650

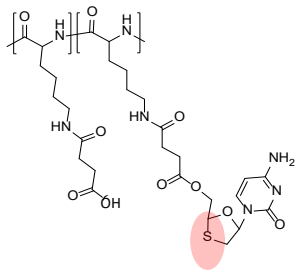
Drug Loading Quantification of Prodrugs



Poly L-lysine succinylated (PLS)



Lamivudine (LAM)



PLS-LAM

Sample	%S	%N
Poly L-lysine succinylated	0.46	12.17
Lamivudine	14.17	18.44
PLS-LAM	1.37	12.58

$$\%WT_{LAM} = \frac{\%S_{prodrug} - \%S_{PLS}}{\%S_{LAM} - \%S_{PLS}} \times 100\%$$

$$\%\boldsymbol{WT_{LAM}} = \mathbf{6.6 \pm 0.4\%}$$

$$\%WT_{LAM} = \frac{\%N_{prodrug} - \%N_{PLS}}{\%N_{LAM} - \%N_{PLS}} \times 100\%$$

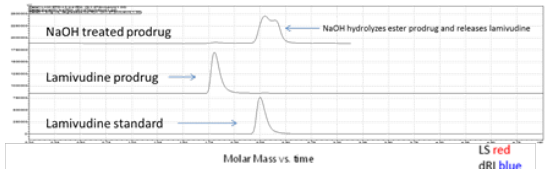
$$\%\boldsymbol{WT_{LAM}} = \mathbf{7.0 \pm 0.9\%}$$

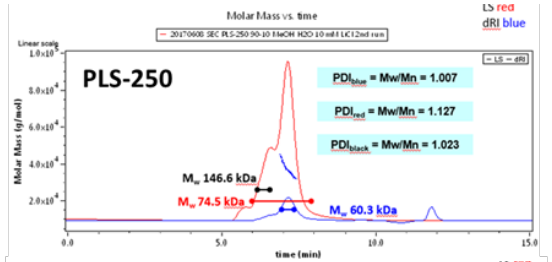
Orthogonal methods comparison

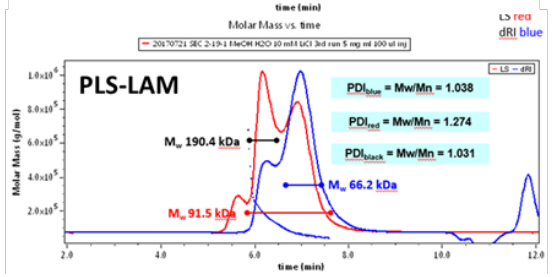
Prodrug	CHNS elemental analysis	CHN elemental analysis	Hydrolysis & RP-HPLC	SEC-MALS
PLS-LAM	6.6 ± 0.4%	7.0 ± 0.9%	6.7 ± 0.1% 7.4 ± 0.1%	5.7 ± 3.8%

Advantages

- ✓ Robust: no method development required
- ✓ Fast: approximately 5 min/sample in CHN mode; 7 min/sample in CHNS mode
- ✓ ~2 mg sample (powder) needed
- ✓ Accurate: sample-to-sample consistency, validated by other methods



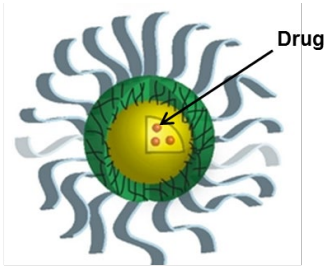




New method to quantitate polymer-bound drug

Hu Y, Stevens DM, Man S, Crist RM, Clogston JD. Total drug quantification in prodrugs using an automated elemental analyzer. Drug Deliv Transl Res. 2019 Dec;9(6):1057-1066.

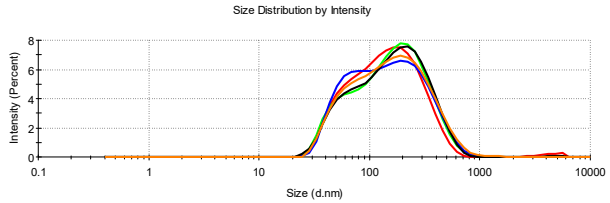
Drug Stability by AF4-DLS-HPLC



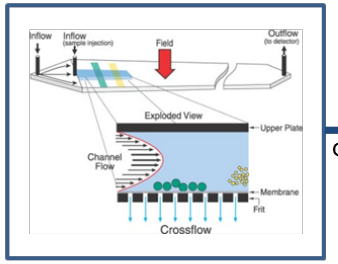
Polymeric micelles

Batch-mode DLS

Where is the drug located?



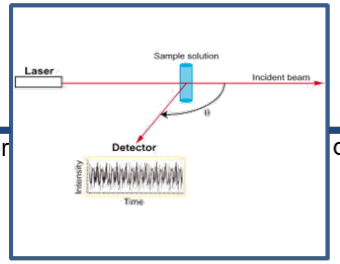
Asymmetric-flow field-flow fractionation (AF4)



Separates NP, plasma

in-line detector

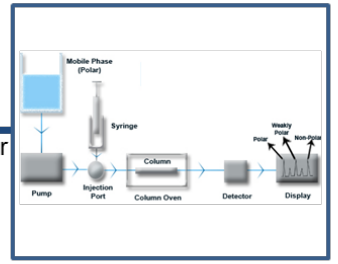
Dynamic light scattering (DLS)



Measures hydrodynamic size

off-line detector

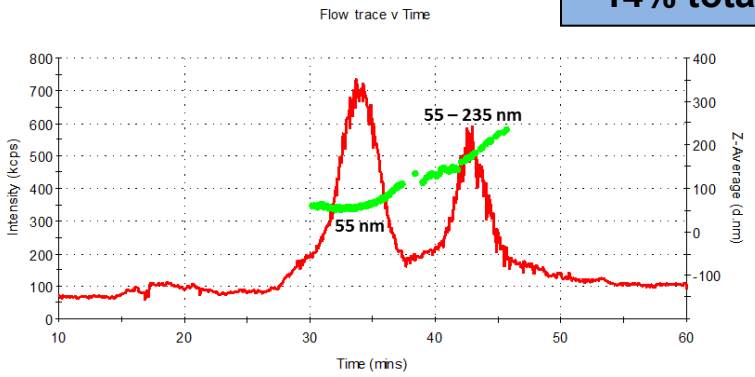
Reversed-phase High performance liquid chromatography (RP-HPLC)



Measures drug concentration

Flow-mode DLS

14% total Drug Recovered

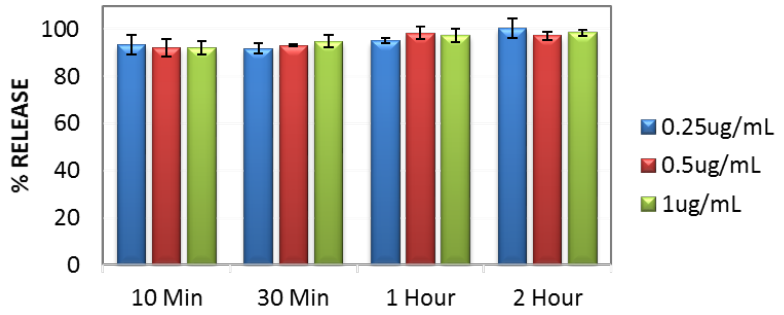


- **AF4 membrane passivated with BSA**

- **Drug partitions to BSA; has more affinity for protein than NP**

NCL359 in Plasma

85-100% released Drug



Findings are similar to stable isotope method.

A new method to assess drug stability.

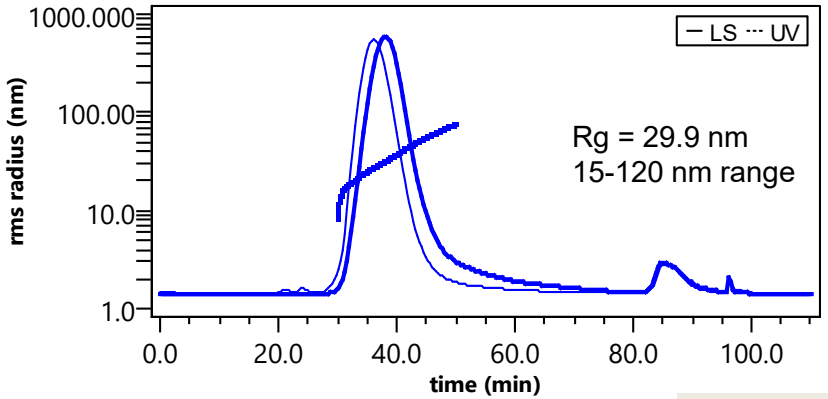
Tested on several micellar and liposomal formulations with consistent results.

Hu Y, Crist RM, Clogston JD. Anal Bioanal Chem. 2020 Jan;412(2):425-438.
Skoczen SL, Stern ST. Methods in Molecular Biology. Vol. 1628, 2018, Humana Press, New York, NY. p. 223-239.

AF4 Analysis of Lipid Nanoparticles (LNPs)

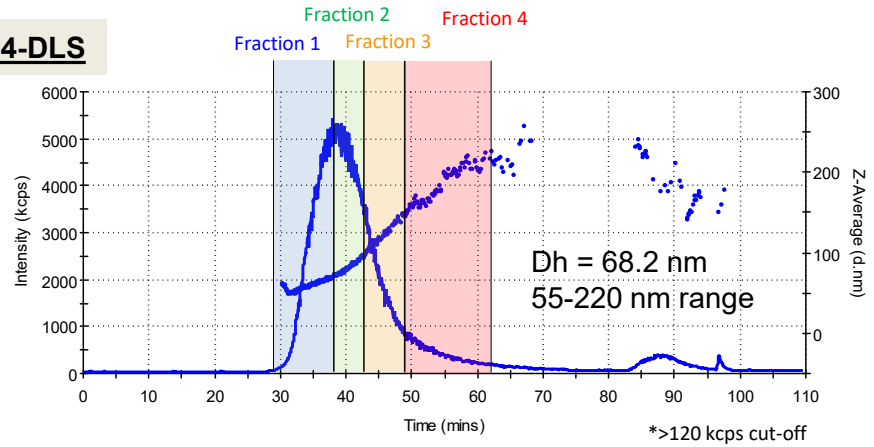
Aim: Develop an AF4 method to examine the heterogeneity related to the density and composition of LNPs (Lipid Nanoparticles) for mRNA Delivery

AF4-UV-MALS



Start Time (min)	End Time (min)	Duration (min)	Mode	Vx Start (mL/min)	Vx End (mL/min)
0.00	2.00	2.00	Elution	1.00	1.00
2.00	4.00	2.00	Focus		
4.00	9.00	5.00	Focus + Inject		
9.00	19.00	10.00	Focus		
19.00	29.00	10.00	Elution	1.00	1.00
29.00	31.00	2.00	Elution	1.00	0.50
31.00	81.00	50.00	Elution	0.50	0.50
81.00	83.00	2.00	Elution	0.50	0.00
83.00	95.00	12.00	Elution	0.00	0.00
95.00	97.00	2.00	Elution + Inject	0.00	0.00
97.00	107.00	10.00	Elution	0.00	0.00

AF4-DLS



AF4 method developed for LNPs which allows for the collection of peak fractions for further analysis.

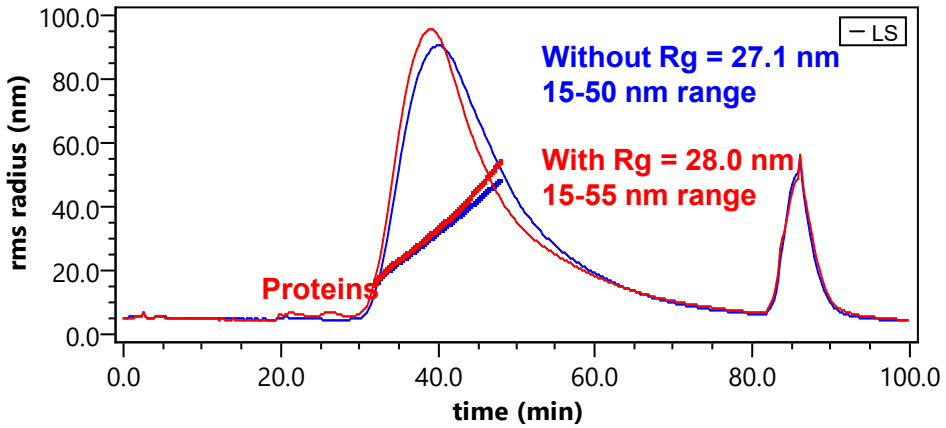
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Total
Time (min)	29 – 38.5	38.5 – 44	44 – 50	50 – 63	-
Volume (mL)	8.99	5.67	6.01	12.32	-
Dh range (nm)	50 – 83	83 – 127	127 – 177	177 – 250	-
	%Recovery*				
Lipid #1	53.8	17.2	ND	ND	71.0
Chol	49.3	22.8	3.0	0.8	75.9
Lipid #2	52.1	29.6	5.2	1.4	88.3
DSPC	66.4	29.3	4.6	ND	100.3
mRNA	33.6	15.7	3.6	3.0	55.9

* Normalized to injected amount
ND = Not Detected

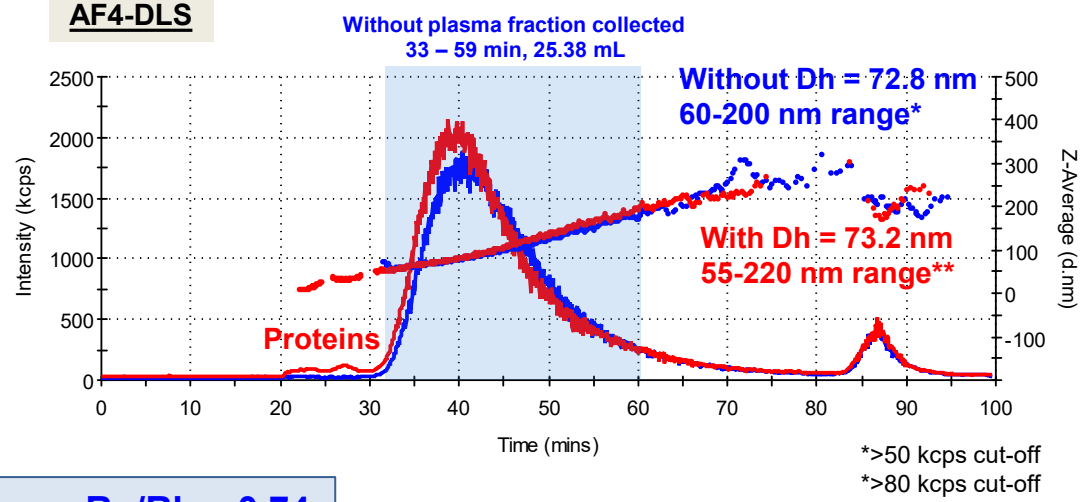
Assessing Protein Binding by AF4

Aim: Assess protein binding using AF4

AF4-UV-MALS



AF4-DLS



*>50 kcps cut-off
*>80 kcps cut-off

Shape factor $\rho_{without}$ = Rg/Rh = 0.74
Shape factor ρ_{with} = Rg/Rh = 0.77

Collected fractions also analyzed for zeta potential and particles per mL concentration

Zeta Potential	ZP (mV)
AF4 fraction (n=2)	-2.6

Total recovered particles: **6.86E+10**
Total injected particles: **6.34E+10**
Particle Recovery% = **108 ± 3%**

Run #	Diameter (nm)	Attenuation level (%)	Laser Power (%)	Particle concentration (1/mL)	Particle concentration * fraction volume
-1	98.9	75	93	2.78E+09	7.05E+10
-2	97.8	67	87	2.71E+09	6.88E+10
-3	98.3	67	87	2.62E+09	6.64E+10
Average	**98.3**			**2.70E+09**	**6.86E+10**
SD	**0.6**			**8.04E+07**	**2.04E+09**

PCC Challenges

The analytical techniques described are *ensemble* methods, that is methods which measure the average or bulk properties.

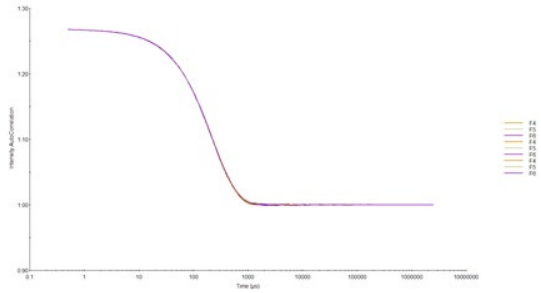
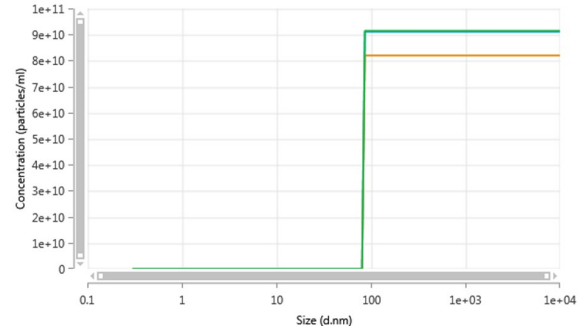
- **For example, dynamic light scattering gives the overall size of the sample but not how many nanoparticles are of a certain size.**
- **Total drug loading determination gives the overall drug concentration. It does not measure, however, how the drug is distributed over the size range.**
- **For drug loaded liposomes, these techniques cannot measure how many liposomes have drug and how many are empty.**
- **Moreover, if they are loaded with drug, what is the extent of drug loading.**

- **To answer these questions, more advanced analytical techniques/methods are needed.**
- **Moreover, as the field advances, so does the complexity of the nanoformulations.**
  - **Analytical methods based on a per particle basis are required.**

PCC Challenges: Assessing Particle Concentration

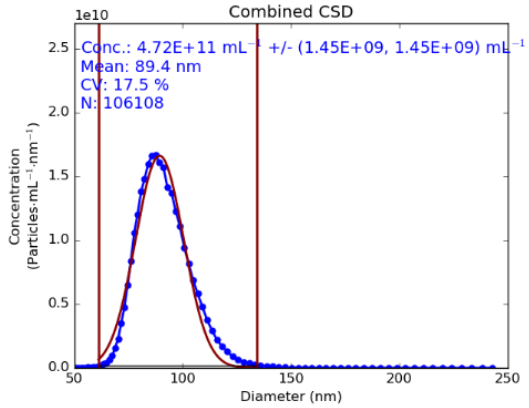
Wyatt DynaPro, Malvern Ultra
Based on light scattering

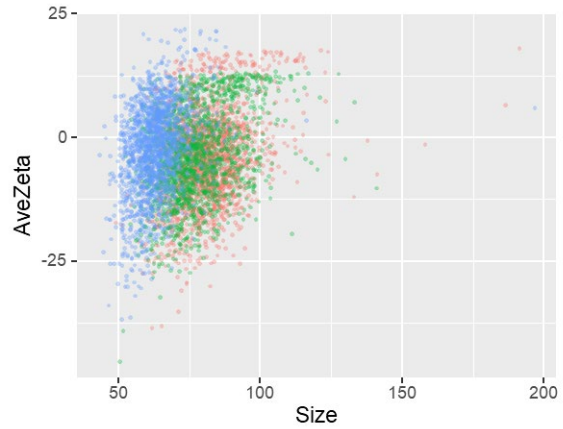


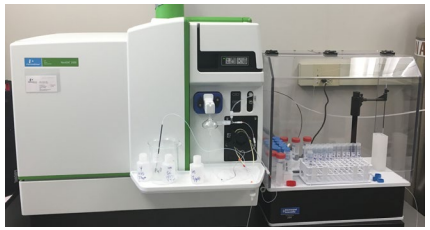
Spectradyne nCS1, Izon qNano
Based on resistive pulse sensing;
lower limit of ~50 nm







PerkinElmer NexION 2000 ICP-MS
Based on single-particle ICP-MS;
metal-containing NPs only



➢ **Need for reference materials for particle concentration**

PCC Challenges: Assessing New Technologies

Questions to be answered:

1. What is the resolving power in terms of a mixture of different size populations?
2. Can the technology measure the relative ratios of non-drug loaded and drug-loaded liposomes?
3. Can the technology measure the amount of drugs loaded in the liposomes and the load distribution?
4. Can the technology measure the amount of free drug?
5. Can the technology measure the release of drug in plasma?

Imperial College London has developed a technology named *single particle automated Raman trapping analysis* (SPARTA), which is a comprehensive nanoparticle analysis platform *based on Raman spectroscopy* providing *simultaneous size, composition and functionalization analysis* as well as allowing monitoring of dynamic reactions occurring at the surface of individual particles.

The technology enables fast, high throughput, routine analysis of individual nanoparticles in solution *without any need for particle labelling or modification*.

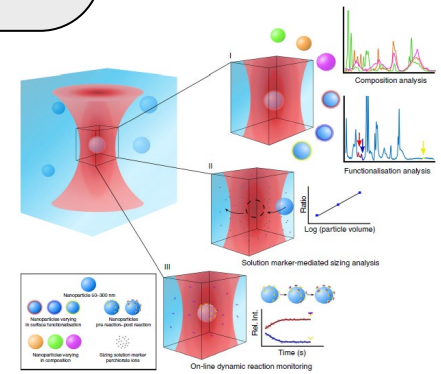


Figure: Nature Communications, 2018, 9, 4256. https://www.imperial.tech/available-technologies/sparta/

Spectradyne's Arc particle analyzer uses microfluidic technology combined with fluorescent imaging to detect the fluorescent signal and measure the *size* of each particle in your formulation. This unique combination yields *phenotyping* and electrically-based particle sizing of each particle as it passes through a nanoconstriction, so that you know the particle type as well as *size* plus *concentration* information.

https://nanoparticleanalyzer.com/products_arc.php?orig_page=index.php

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Hannah Newton


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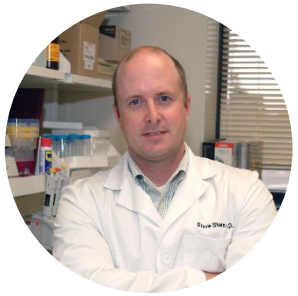
Stephan Stern


Alison Vermilya


Teagan Ware


Jie Xu

Contact Information

Contact Info:
Jeffrey D. Clogston
(301) 846-1388
clogstonj@mail.nih.gov

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Advanced Technology Research Facility
8560 Progress Drive
Frederick, MD 21701

Phone: 301-846-6939
Fax: 301-846-6399
Email: ncl@mail.nih.gov

Web: http://ncl.cancer.gov